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学位授与の題目	Studies on ferredoxin-NADP+ oxidoreductase from the thermophilic cyanobacterium, Synechococcus elongatus(好熱性ラン色細胞 Synechococcus elongatus の ferredoxin-NADP+ oxidoreductase (FNR) に関する研究)
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学位論文要旨

Abstract

The *petH* gene, encoding ferredoxin-NADP$^+$ oxidoreductase (FNR), was isolated from the thermophilic cyanobacterium *Synechococcus elongatus*. The *petH* gene product consisted of 386 amino acids, and contained the unique domain in cyanobacterial FNR at the N-terminal region, which showed sequence similarity to CpcD-phycobilisome linker polypeptide. The deduced molecular mass of *petH* gene was approximately 43 kDa. *S. elongatus* FNR protein was purified homogeneously in a form of 45 kDa retaining CpcD-like domain using protease inhibitors, and the activity of FNR of 45 kDa form was nearly identical to that of the 34 kDa form. *S. elongatus* FNR was tolerant to proteolytic cleavage when FNR was associated with phycobiliproteins. *S. elongatus* FNR was also purified in the form associating with phycocyanin by repetitive ion-exchange chromatography.

The petH gene of *S. elongatus* was expressed in *Escherichia coli*. The catalyically active and full-length form of *S. elongatus* FNR was expressed in the bacterial cells. The *petH* gene was modified to truncate first 89 amino acids, corresponding to CpcD-like domain, and transformed to the bacterial cells. FNR without CpcD-like domain was expressed in the active form, and homogeneously purified.

Three forms of *S. elongatus* FNR, FNR retaining CpcD-like domain, FNR lacking CpcD-like domain and FNR associating with phycocyanin, were purified from *S. elongatus*, and may reflect the variety of localization and function of cyanobacterial FNR in vivo.

Introduction

Ferredoxin-$NADP^+$ oxidoreductase (FNR, EC 1.18.1.2.) is an enzyme containing an FAD as an electron-transfer component and catalyzes electron transfer between ferredoxin and $NADP^+$/NADPH. The mature FNR proteins, purified and characterized from higher plants, show the molecular masses in a range of 33 kDa to 36 kDa. Cyanobacterial FNR proteins, having the molecular masses in a range of 31.5-36 kDa, are remarkably homologous to higher plant FNRs with FAD- and $NADP^+$.

In spite of the similarity in structural and biochemical features of these FNRs, three cyanobacterial *petH* genes (*Synechococcus* PCC 7002, *Anabaena variabilis* PCC7119, *Synechocystis* PCC6803) encode a protein being composed of three domains with a molecular mass of about 45 kDa. The first, N-terminal domain is unique in cyanobacteria and similar to the 9-kDa phycocyanin-associated linker polypeptide CpcD. The N-terminal domain of cyanobacterial FNR is considered to function as anchor of FNR onto phycobilisome. The two domains of C-terminal side correspond to the FAD- and NADP-binding domains of higher plant FNR protein. It has been reported that many cyanobacterial phycobilisomes contain substoichiometric amount of polypeptide with masses of approximately 45-50 kDa (Yamanaka et al. 1978, Tandeau de Marsac and Cohen-Bazire 1997). However, it is remained to be explicit whether the FNR with CpcD-like domain is a dominant form in living cells, and whether cyanobacterial FNR is proteolytically cleaved when released from phycobilisome during the purification.

I report in this thesis the cloning and sequencing of *petH* gene from a thermophilic cyanobacterium, *Synechococcus elongtus*, and the purification of *S. elongatus* FNR protein as the 45 kDa form retained CpcD-like domain. FNR associating with phycobilisome was also purified from *S. elongatus*. The state of cyanobacterial FNR *in vivo* is also discussed in this thesis.

Results and Discussion

The *petH* gene, encoding ferredoxin-$NADP^+$ oxidoreductase (FNR), was isolated from the thermophilic cyanobacterium *Synechococcus elongatus*. The *petH* gene product consisted of 386 amino acids, and contained the unique domain in cyanobacterial FNR at the N-terminal region, which showed sequence similarity to CpcD-phycobilisome linker polypeptide. Two domains at C-terminus correspond to the FAD- and NADP-binding domains of higher plant FNR protein, which composed the catalytic domains of the enzyme.

The regions connecting the CpcD-like domain with the C-terminal domains of *S. elongatus* FNR showed features typical for 'PEST' site, which was the putative target site for proteolytic cleavages of proteins. These results suggested that CpcD-like domain of *S. elongatus* FNR was connected by a hinge region with the catalytic domains at the C-terminus, and that the region connecting CpcD-like domain and FAD-binding domain of *S. elongatus* FNR was sensitive to proteolytic cleavage.

S. elongatus FNR protein was purified homogeneously in a form of 45 kDa retaining CpcD-like domain using protease inhibitors (Fig.1), and the activity of FNR of 45 kDa form was nearly identical to that of the 34 kDa form. *S. elongatus* FNR was tolerant to proteolytic cleavage when FNR was associated with phycobiliproteins. But when FNR was separated from phycobiliproteins in the absence of protease inhibitors,

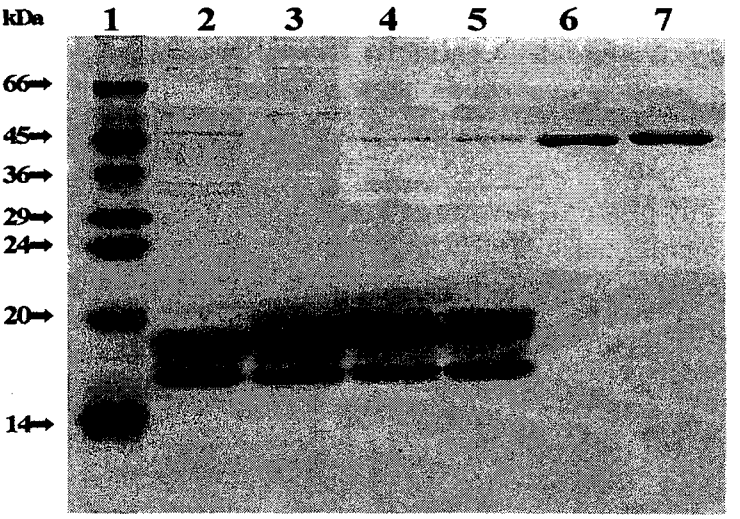

Figure 1: Purification of *S. elongatus* FNR with protease inhibitors. Sample corresponding 5mg protein in lane 2-4 and 5mU DCPIP activity in lane 6, 7 was loaded. The gel was stained with Coomassie brilliant blue R-250. Lane 1: marker, Lane 2: crude extract, Lane 3: 30-40% ammonium sulfate fraction, Lane 4: DEAE-cellulose chromatography, Lane 5: TOYOPEARL HW50S gel filtration chromatography, Lane 6: hydroxyapatite chromatography, Lane 7: Superose 12 gel filtration chromatography.

FNR was proteolyically cleaved between CpcD-like domain and FAD-binding domain to produce a form of FNR with 34 kDa.

The apparent molecular mass of S. elongatus FNR on SDS-PAGE was 45 kDa in the fraction purified by salting out and DEAE-cellulose chromatography. On the other hand, the apparent molecular mass of *S. elongatus* FNR on TOYOPEARL HW50S gel filtration chromatography was ca. 78 kDa. To remove phycobiliproteins from FNR fractions, The fraction showing DCPIP reduction activity was collected, and subjected to mono Q chromatography. The molecular mass of FNR on SDS-PAGE was 45 kDa in a sample purified by repetitive mono Q chromatography, indicating that FNR retained CpcD-like domain. Polypeptitdes with molecular mass of ca. 20 kDa and 16 kDa on SDS-PAGE was also observed in the fraction. These polypeptide were considered to be subunits of phycocyanin because the fraction showed absorption maximum at 615 nm, typical for phycocyanin from phylogenetically diverse cyanobacterial strains. These results suggest that *S. elongatus* FNR of 45 kDa form is associated with phycobcyanin via CpcD-like domain, even after the disassembly of phycobilisome.

The *petH* gene of *S. elongatus* was expressed in *Escherichia coli* .The catalyically active and full-length form of S. *elongatus* FNR was expressed in the bacterial cells. But, FNR was cleaved at the region between CpcD-like domain and FAD-binding domain during an ion-exchange chromatography and produce a form with 34 kDa, nearly identical with that of the form cleaved during the purification from *S. elongatus*. The *petH* gene, modified to truncate first 89 amino acids corresponding to CpcD-like domain, was also transformed to the bacterial cells. FNR without CpcD-like domain was expressed in the active form, and homogeneously purified. The specific activity of FNR without CpcD-like domain was higher than that of FNR retaining CpcD-like domain.

Three forms of *S. elongatus* FNR, FNR retaining CpcD-like domain, FNR lacking

CpcD-like domain and FNR associating with phycocyanin were purified from *S. elongtus*, and may reflect the variety of localization and function of cyanobacterial FNR in vivo.

学位論文審査結果の要旨

これまでに数種の cyanobacteia から *petH* gene が単離され，その産物の性質が明らかにされてきた。すなわち *petH* gene 産物は，3 つの domains (CpcD-like domain と 2 つの catalytic domains) からなる 45kDa の酵素（ferredoxin-$NADP^+$ oxidoreductase）で，光化学系 I から供給される電子を ferredoxin を介して受け取り，$NADP^+$ に伝達する機能を持つ。しかしこれまで実際に精製されてた酵素タンパク質は予想されるものより小さく，CpcD-like domain を失った大きさ（34kDa）のものであった。

本論文では，好熱性 cyanobacterium，*Synechocaccus elongatus* から新たに *petH* gene を単離し，塩基配列を決定し，さらに内在性の蛋白質分解酵素の作用を押さえるため，阻害剤共存下で本来の 45kDa の酵素，CpcD-like domain を失った truncated 酵素および cyanobacteria 特有の phycobiliproteins と結合した蛋白質分解酵素によって分解されにくい酵素複合体の 3 種の精製に成功したことを報告した。また，45kDa および 34kDa 酵素の大腸菌による大量発現系の構築と cyanobacterium の細胞中では 45kDa の酵素が phycobilisome に結合した状態で機能するとの結論を導き出した。

今後の研究に発展が期待されるが，cyanobacteria における *petH* gene の新たな知見を得たことは重要である。よって本論文は博士（理学）を授与するに値すると判断した。